Effective conversion of irinotecan to SN-38 after intratumoral drug delivery to an intracranial murine glioma model in vivo

Laboratory investigation

WELJUN WANG, M.D.,1 ALEX GHANDI, PH.D.,2 LEONARD LIEBES, PH.D.,2 STAN G. LOUIE, PHARM.D.,3 FLORENCE M. HOFMAN, PH.D.,4 AXEL H. SCHÖNTHAL, PH.D.,5 AND THOMAS C. CHEN, M.D., PH.D.1,4

Departments of 1Neurosurgery, 4Pharmacy, 3Pathology, and 5Molecular Microbiology and Immunology, University of Southern California, Los Angeles, California; and 2New York University Cancer Institute, New York, New York

Object. Irinotecan (CPT-11), a topoisomerase I inhibitor, is a cytotoxic agent with activity against malignant gliomas and other tumors. After systemic delivery, CPT-11 is converted to its active metabolite, SN-38, which displays significantly higher cytotoxic potency. However, the achievement of therapeutically effective plasma levels of CPT-11 and SN-38 is seriously complicated by variables that affect drug metabolism in the liver. Thus the capacity of CPT-11 to be converted to the active SN38 intratumorally in gliomas was addressed.

Methods. For in vitro studies, 2 glioma cell lines, U87 and U251, were tested to determine the cytotoxic effects of CPT-11 and SN-38 in a dose-dependent manner. In vivo studies were performed by implanting U87 intracranially into athymic/nude mice. For a period of 2 weeks, SN-38, CPT-11, or vehicle was administered intratumorally by means of an osmotic minipump. One series of experiments measured the presence of SN-38 or CPT-11 in the tumor and surrounding brain tissues after 2 weeks’ exposure to the drug. In a second series of experiments, after 2 weeks’ exposure to the drug, the animals were maintained, in the absence of drug, until death. The survival curves were then calculated.

Results. The results show that the animals that had CPT-11 delivered intratumorally by the minipump expressed SN-38 in vivo. Furthermore, both CPT-11 and SN-38 accumulated at higher levels in tumor tissues compared with uninvolved brain. Intratumoral delivery of CPT-11 or SN-38 extended the average survival time of tumor-bearing animals from 22 days to 46 and 65 days, respectively.

Conclusions. These results demonstrate that intratumorally administered CPT-11 can be effectively converted to SN-38 and this method of drug delivery is effective in extending the survival time of animals bearing malignant gliomas. (DOI: 10.3171/2010.2.JNS09719)

Key Words • glioma • CPT-11 • SN-38 • irinotecan • intratumoral delivery • carboxylesterase

Irinotecan (CPT-11), a topoisomerase I inhibitor, is primarily used in the treatment of solid tumors, particularly in combination with other chemotherapy agents. Its antitumor activity has also been evaluated in patients with malignant gliomas, where combination therapy together with temozolomide or bevacizumab has resulted in encouraging results. However, dose-limiting toxicities associated with systemic CPT-11 therapy, primarily diarrhea and neutropenia, impede optimized dosing and require careful management.1,5,13,16,19,22

In current treatment regimens, CPT-11 is administered intravenously and is extensively metabolized in the liver. The critical metabolite, SN-38, is generated through carboxylesterase metabolism. Plasma levels of SN-38 are considerably lower than the corresponding CPT-11 plasma concentrations.23 However, SN-38 is up to 1000-fold more potent than CPT-11 as an inhibitor of topoisomerase,
which correlates with anticancer activity. Therefore SN-38 represents the critically active component of this therapeutic regimen.\textsuperscript{9,10} Inactivation of SN-38 is accomplished via glucuronidation catalyzed by uridine 5'diphospho-glucuronyltransferase 1A1 (UGT1A1). Patients who express certain genetic polymorphisms in the UGT1A1 gene must receive reduced doses of CPT-11, otherwise SN-38 may accumulate to substantially higher levels and cause life-threatening toxicities.\textsuperscript{2,6,9,11} Another route of CPT-11 may accumulate to substantially higher levels and cause life-threatening toxicities.\textsuperscript{2,6,9,11} Another route of CPT-11 clearance involves hepatic metabolism using CYP3A4 or CYP3A5.\textsuperscript{20}

The metabolic clearance is further complicated by the fact that coadministration with enzyme-inducing antiepileptic drugs (EIAEDs), such as phenytoin, phenobarbital, or carbamazepine, can induce increases in enzyme expression. This is a major obstacle for CPT-11 therapy in brain tumor patients requiring coadministration of enzyme-inducing AEDs, which can accelerate clearance of CPT-11 and therefore substantially reduce systemic exposure to the drug and its active metabolite.\textsuperscript{11,15} For patients with brain tumors, discontinuation of enzyme-inducing AED treatment is an option; however, at least 4 weeks is needed before levels of CYP activity reach baseline levels. Unfortunately, suspension of AED therapy greatly increases the risk of uncontrollable seizures in these affected patients.

Although inclusion of CPT-11 in treatment regimens for malignant gliomas has yielded encouraging results, the above-mentioned complications can make it difficult to adjust dosage for optimal anticancer efficacy. We had previously demonstrated that CPT-11 may be converted to SN-38 in vitro by malignant glioma cells, showing that direct intratumoral conversion of CPT-11 to SN-38 may be a feasible mechanism for bypassing hepatic metabolism and the complications of concomitant anticonvulsant therapy.\textsuperscript{4} In support of this concept, several laboratories have now used various animal models to investigate the possibility of direct intratumoral delivery of CPT-11.\textsuperscript{10,12,14} which would be expected to greatly reduce fluctuations. While this approach has demonstrated antitumor activity of intratumorally delivered CPT-11, it has remained unclear whether any SN-38 is generated under such conditions. We therefore investigated this issue using intratumoral delivery of CPT-11 in a murine brain tumor model, and we found that significant levels of SN-38 are generated under such in vivo treatment conditions. Our findings indicate that glioma cells in vivo are able to convert CPT-11 into the more potent metabolite SN-38, providing support for intratumoral delivery of CPT-11 as a means of maximizing therapeutic efficacy and minimizing systemic toxicity.

**Methods**

**Cell Culture**

The human glioblastoma cell lines U87 and U251 were obtained from American Type Culture Collection and maintained in DMEM (Invitrogen) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 0.1 mg/ml streptomycin in a humidified incubator at 37°C and a 5% CO\textsubscript{2} atmosphere.

**Methylthiotetrazole Assay for Drug Cytotoxicity in Vitro**

Cells were seeded in 96-well culture plates at a cellular density of 1.5 × 10\textsuperscript{4} cells/well and cultured overnight. The unattached cells were then removed and new medium containing various drug concentrations were added. Cells were then incubated for an additional 48 hours, after which time 20 μl of 5 mg/ml methylthiotetrazole (MTT, Sigma) solution was added into each well for 4 hours. Subsequently, the medium was removed and 150 μl dimethyl sulfoxide (DMSO, Sigma-Aldrich) was added to liberate the tetrazolium crystals. The absorbance was determined on a Phelo 96-well plate reader at an optical density of 490 nm.

**Intratumoral Drug Delivery**

The murine tumor model involved 2 parts. First, the human tumor cells were implanted into the animals; then after 10 days the pumps were also implanted into these animals. All animal experiments were approved by the institutional animal care and use committee. The mice used in these studies were athymic/nude, male, 6–8 weeks old (Harlan) and were maintained in a pathogen-free environment throughout the experiment. Tumor cells (200,000 U87 glioblastoma cells per mouse) were implanted stereotactically into the right frontal lobe of the mice. Ten days after tumor cell implantation, a miniature infusion pump (ALZET Osmotic Pumps, DURECT Corporation) was implanted subcutaneously on the back of each animal and connected to an intratumoral infusion catheter via a soft plastic tube. This setup did not impede the movement or normal behavior of the animals. The pumps were prefilled with PBS, CPT-11 (8 mg/kg/d), or SN-38 (100 μg/kg/d) and provided continuous delivery over the course of 2 weeks.

**Survival Curves**

To determine the therapeutic benefit of intratumoral drug delivery, the tumor-bearing animals were administered the drug for 2 weeks by means of the infusion pump. These pumps are able to hold only 2 weeks' worth of drug; thus after 2 weeks the infusion pump was no longer supplying drug. The survival of these animals was then monitored, and survival curves were calculated using the Kaplan-Meier method.

**Biodistribution of Intratumorally Delivered Drugs**

After the 2-week drug delivery period was completed, the animals were anesthetized and killed by cervical dislocation. The brain tissues were then analyzed for the amount of drug available in the different parts of the brain. More specifically, the biodistribution of CPT-11 and SN-38 was assessed in 3 lobes of the brain (frontal, parietal, and cerebellum), as well as in tumor tissue; the tumor tissue was separated from the normal brain tissue and weighed before further processing. Tissue extracts were prepared and drug concentrations were determined by HPLC.

**The HPLC Quantification of CPT-11 and SN-38**

To determine the concentration of CPT-11 and SN-38 in cultured cells in vitro and in tissues from drug-treat-
Direct intratumoral infusion of CPT-11

ed animals in vivo, a validated HPLC method was employed. In brief, approximately 2–3 × 10^6 U87 cells were lysed with 60% (v/v) methanol/water as described. The cellular extract was then centrifuged, and the supernatant was removed and transferred to a sterile vial. Methanol was evaporated under a steady stream of nitrogen, and the evaporated residue was dissolved in H_2O. The intracellular levels of CPT-11 and SN38 were analyzed using an isocratic HPLC system that consisted of a Knauer 64 HPLC pump, a Waters 717 Plus autosampler, an ABI Analytical 980 fluorescence detector, and an Axxiom 747 data analysis/control system. The chromatographic separation used a Phenosphere analytical column (150 × 4.6 mm) packed with 3-μm, 80-Å Hypersil ODS2 (Phenomenex). The column was eluted at a flow rate of 1.0 ml/minute with a mobile phase consisting of acetonitrile, 25 mM potassium phosphate, pH 4.0, triethylamine (24/72/4, v/v/v). The column effluent was monitored using excitation at 355 nm and an emission wavelength of 520 nm.

Statistical Analysis

All nonparametric data were analyzed using the Student t-test to calculate the significance values; a probability value < 0.05 was considered statistically significant.

Results

In Vitro Cytotoxicity of CPT-11 and SN-38

Human glioblastoma U87 cells were incubated with increasing concentrations of CPT-11 or SN-38 for 48 hours, and cell survival was determined by MTT assay. The results (Fig. 1) demonstrated a similar concentration-dependent reduction in cell viability of U87 and U251 cells in the presence of either drug. Both U251, p53 mutant, and U87, p53 wildtype, were used to show that this drug activity was not restricted to 1 cell line or p53 status. As expected from previous studies with these compounds, SN-38 was substantially more cytotoxic than CPT-11, with an IC_{50} of 750 nM versus 85 µM in U87, and 10 nM versus 10 µM in U251, respectively. These in vitro dose-response curves are consistent with other studies.\(^\text{1,4}\)

In Vitro Conversion of CPT-11 to SN-38 by U87 Cells

We next determined whether cultured U87 cells would be able to generate SN-38 from CPT-11. The U87 cells were incubated with 100 µM CPT-11 for 48 hours, and then cell extracts were prepared and analyzed by HPLC. As shown in Fig. 2, the presence of SN-38 was clearly detected, indicating the ability of these cells to convert CPT-11 to its more toxic metabolite SN-38. Having confirmed this metabolic conversion in vitro, we moved on to investigate whether U87 cells could display this activity in an orthotopic tumor model in vivo as well.

Effect of CPT-11 and SN-38 on Glioblastoma Growth in Vivo

We next investigated whether direct intratumoral delivery of either CPT-11 or SN-38 would display comparable therapeutic efficacy in an orthotopic U87 brain tumor model in mice. We used U87 glioblastoma cells in all in vivo studies. Intratumoral drug delivery into the cranium was accomplished with the use of an ALZET mini-pump, which was subcutaneously implanted on the back of tumor-bearing animals and connected to an intratumoral infusion needle. The pumps were prefilled with either PBS (vehicle control), CPT-11, or SN-38 and continuously released through intratumoral delivery over the course of 14 days. Therapeutic efficacy of this delivery system was characterized in 2 ways: 1) by measuring tumor weight at a specific time after treatment, and 2) by determining the survival of tumor-bearing animals.
In the first approach, all animals were killed 2 weeks after the onset of drug treatment, by that time the mini-pumps had finished their course of drug delivery. The skulls were opened, brains removed, and the tumor tissue dissected out of the brain. This was possible because the intracranial growth of U87 cells in vivo takes place via spherical expansion without substantial infiltration of tumor cells into the surrounding tissues. Thus it was relatively straightforward to distinguish tumor tissues from healthy tissue. All tumors were weighed, and the results are summarized in Table 1. Treatment with CPT-11 reduced tumor weight to approximately 50%, whereas treatment with SN-38 showed a stronger effect and reduced tumor weight to approximately 30% of the weight of PBS-treated control tumors. The differences between control and drug-treated tumor weights were statistically significant (p < 0.05), but no statistical difference between CPT-11 and SN-38 treated tumors was apparent (p = 0.144). As a further control, CPT-11 was injected intravenously into tumor-bearing animals. In these animals, neither CPT-11 nor SN-38 was detected in either the tumor or contralateral brain (data not shown).

In the second approach, to compare therapeutic efficacy of CPT-11 and SN-38 in the intracranial tumor model, all animals were monitored for length of survival after the 2-week drug delivery period. Survival was plotted using Kaplan-Meier curves (Fig. 2). These data show that the average length of survival of animals receiving CPT-11 treatment was significantly (p < 0.0001) prolonged and was more than double that of the PBS-treated controls. The average extension of survival of SN-38 treated animals was also significant (p < 0.0001), and these mice survived nearly 4 times longer than control animals. These results also illustrate the significant differences in survival times between animals treated with SN-38 and those treated with CPT-11 (p < 0.0001). These results demonstrate the therapeutic benefit of continuously delivering CPT-11 or SN-38 intratumorally via osmotic intratumoral delivery over the course of 2 weeks.

Conversion of CPT-11 to SN-38 After Intratumoral Infusion

Metabolic conversion of CPT-11 to its active metabolite SN-38 after intratumoral delivery has not been demonstrated before. It was unclear, therefore, whether therapeutic efficacy of CPT-11 in our tumor model was due to the prodrug itself, or perhaps a result of its conversion to the highly active metabolite, SN-38. As demonstrated in Fig. 2 and in previous studies, we showed that U87 cells were able to metabolically convert CPT-11 to SN38 in vitro. Therefore we investigated whether tumor tissue could accomplish this task in vivo without the involvement of liver enzymes.

We first measured the amount of SN-38 in tumor tissues from animals after 2 weeks of intratumoral infusion of either PBS, CPT-11 (8 mg/kg/d), or SN-38 (0.1 mg/kg/d). As shown in Fig. 3, SN-38 could be readily detected in tumors from animals treated with CPT-11, and the average concentration was 11.3 pmol per mm³ of tissue. In comparison, tumors from SN-38–treated animals harbored 18.0 pmol/mm³. No SN-38 was detected in PBS-treated tissues, as expected.

We then determined the biodistribution of SN-38 within intracranial tissues by comparing SN-38 concentrations in tumor tissue, cerebellum, and residual brain tissues after treatment of animals as above. As shown in Fig. 4, the highest concentrations of SN-38 were measured in tumor tissues, and this result was obtained whether animals were treated with SN-38 or with CPT-11, although the overall amount of SN-38 was higher in tumors from SN-38-treated animals. In comparison, the amount of SN-38 in cerebellum or residual brain tissue was lower by more than an order of magnitude, and this was true in the case of CPT-11 as well as SN-38 treatment. As expected, tissues from PBS-treated animals did not contain SN-38. These results demonstrate that there are significantly higher SN-38 concentrations in the tumors as compared with normal brain tissues. Furthermore, these data confirm the presence of substantial amounts of SN-38 after the intratumoral delivery of CPT-11.

To gain some insight into the efficiency of metabolic synthesis of SN-38 after intratumoral infusion of CPT-11, we investigated the intracranial biodistribution of both compounds, CPT-11 and SN-38, after intratumoral infusion of CPT-11. As shown in Fig. 5, the concentration of CPT-11 in tumor tissues was several hundred-fold higher

TABLE 1: Mean tumor weights after 2 weeks

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tumor Weight (mg)†</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Comparison w/ CPT-11</td>
<td>Comparison w/ SN-38</td>
</tr>
<tr>
<td>PBS</td>
<td>62.967 ± 2.483</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>CPT-11</td>
<td>30.9 ± 6.035</td>
<td>NA</td>
</tr>
<tr>
<td>SN-38</td>
<td>18.2 ± 1.656</td>
<td>0.144</td>
</tr>
</tbody>
</table>

* NA = not applicable.
† Mean ± SE.
of SN-38 in tissue extracts was quantified by HPLC. Shown is the average amount of SN-38 (mean ± SD; n = 5) in the 3 treatment groups.

than in nontumor tissues. The SN-38 concentrations in tumor tissues were 9-fold higher than in nontumor tissues. The absolute concentration of CPT-11 (> 10,000 ng/ml) in tumor tissues was roughly 250-fold higher than the corresponding absolute concentration of SN-38 (45 ng/ml), which is in line with the large plasma differential of these 2 drugs that has been reported after systemic delivery of CPT-11 in patients or laboratory animals.8

Discussion

Investigations into the therapeutic use of CPT-11 for patients with malignant gliomas, in particular in combination with other anticancer agents, have yielded encouraging results.13 However, systemic delivery of CPT-11 is complicated by genetic factors in the patient as well as by drug interactions with anticonvulsant agents. Both of these parameters may severely influence drug metabolism and thus therapeutic outcome and severity of side effects, such as intractable diarrhea. An in effort to circumvent these difficulties, several laboratories, including ours, have been investigating the feasibility of intratumoral drug delivery. In this regard, nanoliposomal delivery of CPT-11 into the cranium of animals with experimental glioblastoma showed promising therapeutic efficacy.10,14 Direct intrathecal administration of anticancer drugs is also an approach used to circumvent the pharmacological sanctuary imposed by the blood-brain barrier.21

Our study using intratumoral delivery of CPT-11 into the cranium of mice harboring orthotopically implanted U87 glioblastoma cells displayed very substantial therapeutic efficacy by significantly extending the survival times of treated animals. Although these results clearly demonstrate therapeutic efficacy of intratumoral drug delivery, it was unclear whether this outcome was caused by CPT-11 itself or potentially by its much more potent metabolite, SN-38. In those instances where CPT-11 is delivered intravenously, it is well established that the activity of hepatic carboxylesterases, primarily CES2, represents the major enzymatic source for the production of SN-38, and this metabolite mediates the major antitumor activity of CPT-11 therapy. To a lesser extent, the expression of CES2 has also been detected in human tissues other than the liver,24 and also in different types of tumor tissues.25

Based on our findings that human U87 glioblastoma cells in vitro are able to generate SN-38 in response to treatment with CPT-11,4 we investigated whether such cells could accomplish this metabolic conversion in vivo as well. We found that this was indeed the case. Intriguingly, accumulation of the in vivo–generated SN-38 preferentially takes place in the tumor tissue, rather than in normal parts of the brain, and therefore displays desirable biodistribution. The mechanism for this specific uptake is not clear; however the blood-brain barrier associated with tumor tissue and the tumor vasculature itself has been shown to be leakier than blood vessels in the normal brain.3 When compared with intratumoral delivery of SN-38 itself, it becomes clear that the amount and biodistribution of the drug is rather similar in both cases—whether it is delivered as SN-38 or whether it is newly generated in vivo after delivery of CPT-11.

Because it has been reported that systemic administration of CPT-11 in animals in vivo and in patients showed only modest antitumor activity, we tested whether these results were due to insufficient accumulation of drug in the brain.5 Our studies demonstrated that indeed, when CPT-11 is administered intravenously, the drug is not detectable in the intracranial tumor (data not shown), thus suggesting a rationale for the lack of activity with systemic administration and highlighting the therapeutic significance of intratumoral CPT-11 administration.

Taken together, our data demonstrate that intratumoral delivery of CPT-11 into the cranium of animals with orthotopically implanted U87 cells results in the de novo production of SN-38 at levels that preferentially accumulate in the tumor tissue and that are therapeutically effective at prolonging the survival of treated animals. The relatively targeted specificity of SN-38 is particularly important in view of this metabolite’s recognized neurotoxicity7 and indicates that the risk for this side effect may be greatly re-
duced. This assumption is further supported by the lack of seizures in the drug-treated animals in our study. Furthermore, a histological examination of the whole brain did not reveal signs of intracranial hemorrhage (neither adjacent to the tumor nor at the contralateral cerebrum) (data not shown). Because our 2-week delivery schedule represents a relatively short period of drug administration, it will be important in the future to determine whether the highly encouraging therapeutic profile of this schedule can be further improved by using prolonged duration or repeated schedules of drug infusion.

Conclusions

Our results demonstrate for the first time that glioma cells, but not normal brain, have the capability of converting the prodrug CPT-11 into its active metabolite SN-38. These data provide impetus for future studies to examine the role of direct delivery of CPT-11 (via convection-enhanced delivery) into malignant gliomas.

Disclosure

This work was supported by awards from the Connell Foundation, the Department of Neurosurgery, the Sounader Foundation, and the Kriegel Foundation (to T.C.C.), and by National Cancer Institute Grant CA014089-33 (to S.G.L.). The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

Author contributions to the study and manuscript preparation include the following. Conception and design: Chen, Liebes, Hofman. Acquisition of data: Wang, Ghandi, Louie. Reviewed final version of the manuscript and approved it for submission: all authors. Statistical analysis: Ghandi. Administrative/technical/material support: Chen, Wang, Liebes, Hofman, Louie. Schönhalt. Study supervision: Chen, Liebes, Hofman, Schönhalt.

Acknowledgment

The authors appreciate the help from the summer volunteers, Kristen Chen and Nathan Chen.

References


Please include this information when citing this paper: published online April 23, 2010; DOI: 10.3171/2010.2.JNSO09719.

Address correspondence to: Thomas C. Chen, M.D., Ph.D., Department of Neurosurgery, University of Southern California, Los Angeles, California 90033. Email: tcchen@usc.edu.